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Development of solid-phase extraction and methylation procedures to analyse free fatty acids in lipid-rich plant materials

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Objectives

The objective of the present work was to develop a solid-phase extraction-gas chromatography (SPE-GC) method to isolate and measure free fatty acids (FFA) in lipid-rich seeds for immediate use in our investigations on the non-orthodox storage behaviour of numerous tropical seeds, such as *Citrus* and coffee seeds, which generally contain large amounts of lipids. In order to develop a sensitive and reliable method, two SPE reference procedures (1,3) employed in food chemistry were compared using a 100/1 mixture of triolein/heptadecanoic acid. The SPE method providing the best results, together with the fatty acids (FA) methylation procedure were then further refined for decreasing triacylglycerols (TAG) contamination in the FFA fraction to achieve accurate measurement of FFA in lipid-rich seeds. The method was finally compared to the conventional thin layer chromatography (TLC) purification procedure used classically in plant physiology studies and validated in ageing coffee seeds.

Conclusions

The present work describes the step-by-step optimization of a new procedure combining SPE and GC for FFA analysis in lipid-rich seeds (2). After a comparison of two general SPE methods, modification of the elution volume of neutral lipids resulted in a decrease in TAG pollution from about 17 to 12%. Removing the saponification step of the standard procedure ISO-5509 for fatty acid methyl esters (FAME) preparation led to a further decrease in the percentage of FAME deriving from TAG from about 8% to 4%. Finally, percentage contamination was further decreased to 1.5% by reducing the duration of the BF_3-catalyzed methylation reaction to 1 min. When validated in ageing coffee seeds, the new procedure provided results in full agreement with the conventional TLC purification procedure. In addition, it combines the advantages of SPE (rapidity, simplicity and fractionation of high amounts of lipids) and the benefit of a new simplified methylation procedure. Finally, it avoids the selective loss of unsaturated FA encountered with TLC purification.

Method development

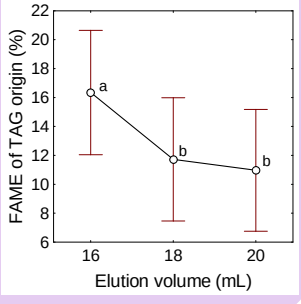
1 SPE reference method comparison

Table 1: Characteristics of the two SPE methods tested for fractionation of the major seed lipid classes: stationary phase, conditioning and loading solvents, elution method and lipids theoretically eluted in Fractions 1 to 3.

Method	M1	M2	Expected lipids
Phase	NH2	Si	
Conditioning	Hexane	Hexane	
Loading	$CHCl_3$	$CHCl_3$	
Fraction 1	16 mL $CHCl_3$/ Isopropanol (2/1)	16 mL Hex/Et_2O (8/2)	Neutral lipids
Fraction 2	16 mL Et_2O/ Acetic acid 2%	16 mL Hex/Et_2O (1/1)	FFA
Fraction 3	16 mL MeOH	16 mL MeOH	Polar lipids

2 Elution volume improvement

Figure 1: Influence of the elution volume used during the first step of the SPE M1 method on TAG pollution in Fraction 2 as detected by GC after fractionation of 100 mg of a 100/1 mixture of triolein / heptadecanoic acid and FA methylation according to the ISO-5509 standard.



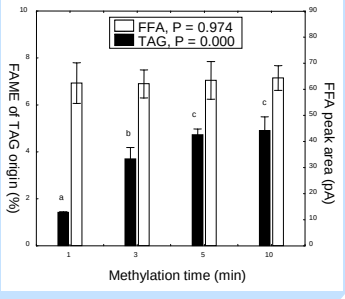
3 Saponification removal

Table 2: Effect of saponification of lipids recovered in Fraction 2 on the amount of FFA and TAG detected by GC after fractionation of 100 mg of a 100/1 mixture of triolein / heptadecanoic acid and BF_3-catalysed FA methylation (3 min).

	FAME of TAG origin (%)	FFA peak area (pA)
With saponification	8.69 ± 1.47	66.0 ± 6.4
Without saponification	3.70 ± 0.52	62.1 ± 5.6
F	30.69	0.63
P	0.005	0.471

4 Methylation time optimization

Figure 2: Influence of the duration of BF_3-catalysed methylation (without saponification) of lipids recovered in Fraction 2 on the amount of FFA and TAG detected by GC after fractionation of 100 mg of a 100/1 mixture of triolein / heptadecanoic acid.



TAG pollution in FFA fraction 17% → 12% → 4% → 1.5%

New SPE-GC method

1-SPE conditions

Table 3: Characteristics of the new SPE-GC method for fractionation of the major seed lipid classes: stationary phase, conditioning and loading solvents, elution method and lipids eluted in Fractions 1 to 3.

New Method		Expected lipids
Phase	Aminopropyl	
Conditioning	Hexane	
Loading	$CHCl_3$	
Fraction 1	18 mL $CHCl_3$/ Isopropanol (2/1)	Neutral lipids
Fraction 2	16 mL Et_2O/ Acetic acid 2%	FFA
Fraction 3	16 mL MeOH	Polar lipids

2-FAME preparation

No saponification
Methylation time MeOH/BF_3 : 1 min

Method validation (coffee + oil palm)

Figure 3: Changes in FFA content (O,●) and viability (□,■) of seeds stored for 0 to 18 months at 15°C under 62% RH. FFA were measured using both the conventional TLC procedure (O) and the new SPE-methylation method (●). After storage, seeds were either directly placed under germination conditions (■) or pre-heated in a 40°C water bath for 30 min prior to sowing (□).

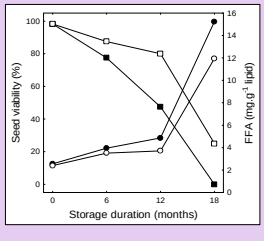
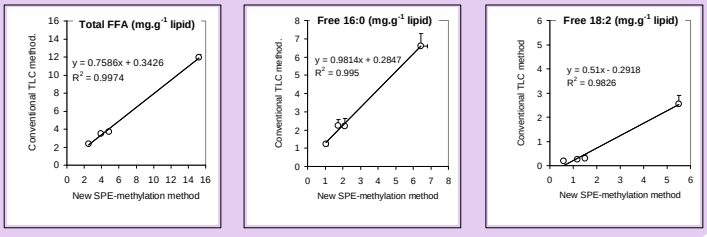


Table 3: Mesocarp content in FFA of various palm genotypes previously characterized for their intrinsic lipase activity. FFA were measured 1h after mesocarp bruising.

Group	FFA (%)
E. guineensis low lipase genotypes	0.6
E. guineensis medium and high lipase introgressed lines	25.8 - 40.2
Elaeis oleifera	2.1

- Full agreement with conventional TLC/on-silica methylation procedure
- Advantages : rapidity, simplicity and fractionation of high amounts of lipids
- Avoids selective loss of unsaturated FA

Figure 4: Analysis of correlations between values obtained with the conventional TLC procedure and the new SPE-methylation method for the total FFA content of seeds and the content in the four major fatty acids: evidence for selective loss of unsaturated FA with the conventional TLC procedure.



References

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